BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/12, C07K 13/00
A61K 39/00, C12N 5/16

A1

(11) International Publication Number: WO 92/03548

(43) International Publication Date: 5 March 1992 (05.03.92)

(21) International Application Number: PCT/EP91/01538

(22) International Filing Date: 13 August 1991 (13.08.91)

(30) Priority data:
90202287.0 27 August 1990 (27.08.90) EP
(34) Countries for which the regional or international application was filed: AT et al.

(71) Applicant *(for all designated States except US):* AKZO N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL).

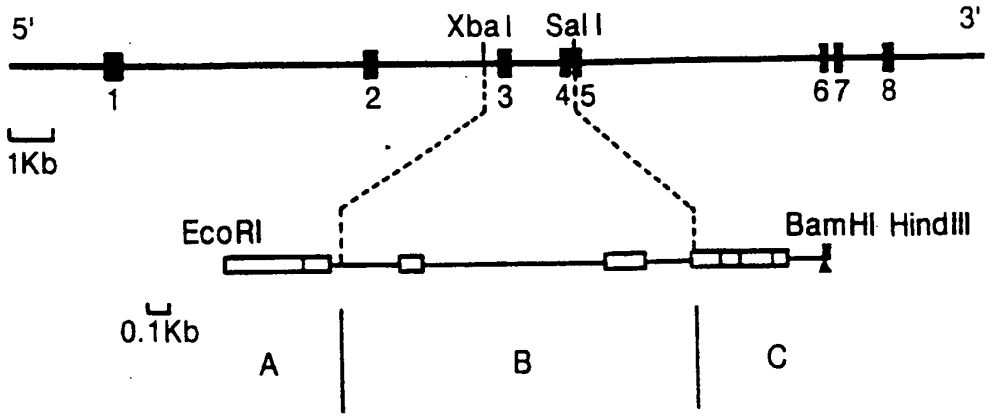
(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : VAN DUIN, Marcel [NL/NL]; Zilverahorn 3, NL-5237 HB 's Hertogenbosch (NL).

(74) Agent: TURNER, Allen, Carey; Postbus 20, NL-5340 BH Oss (NL).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HUMAN ZONA PELLUCIDA PROTEIN ZP3



(57) Abstract

The invention comprises a novel polypeptide or a functional derivative thereof having human ZP3 activity or human ZP3 antigenicity. It also comprises epitopes of said polypeptide, antibodies to the polypeptides or the epitopes, as well as vaccines for contraception and methods for expressing the polypeptides in a suitable host.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE*	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

HUMAN ZONA PELLUCIDA PROTEIN ZP3.

The invention relates to a polypeptide or a functional derivative thereof having human ZP3 activity or having, at least in part, human ZP3 antigenicity.

During the process of fertilization, the first interaction between mammalian gametes is mediated by binding of sperm cells to a species specific receptor on the zona pellucida (ZP) that surrounds the female gamete. The ZP is an extracellular matrix which comprises three glycoproteins, designated ZP1, ZP2 and ZP3, of which ZP3 has been identified as the sperm receptor (reviewed in Wassarman, Development 108, 1-17; 1990).
Numerous in vitro and in vivo studies using both porcine and murine ZP proteins have indicated that ZP3 is an important candidate target antigen in experimental strategies aimed at the development of immuno-contraception (Henderson et al., J. Reprod. Fert. 83, 325-343; 1988 and refs. therein). The recent cloning and characterization of the murine ZP3 cDNA by screening of a mouse ovary cDNA expression library with anti-mouse ZP3 antibodies represents an important step towards this end (Ringuette et al., Developmental Biology 127, 287-295; 1988). Subsequently, ZP3 cDNA probes were used for isolation of the corresponding genomic ZP3 DNA (Chamberlin and Dean, Developmental Biology 131, 207-214, 1988; Kinloch et al., Proc. Natl. Acad. Sci. USA 85, 6409-6413,1989).
The potential of ZP3 for contraception was emphasized by studies that revealed a long term infertility following vaccination of female mice with an oligopeptide derived from the murine ZP3 amino acid sequence (Millar et al., *Science* 246, 935-938; 1989).
For immunocontraception in man, mouse polypeptides are not suitable since the ZP3 sperm receptor is species specific. Moreover, polypeptides from non-human origin may lead to unwanted immune responses in man. Therefore, development of a safe and efficient contraceptive vaccine based on (part of) the human sperm receptor requires information on and availability of the human ZP3 polypeptide.

The isolation of sufficient amounts of human ZP3 polypeptide from female gametes is of course not possible. However, we have succeeded in identifying a human gene and elucidating its sequence. This opens the possibility to produce ZP3 polypeptides and/or ZP3 polypeptide fragments either by recombinant DNA technology or solid phase polypeptide synthesis.

The invention therefore comprises a novel DNA-molecule coding for at least a part of human ZP3 polypeptide. Said DNA-molecule comprises at least a part of the sequence shown in Fig. 2.

The invention also comprises a polypeptide coded for by at least part of the above-mentioned DNA-molecule. Said polypeptide may comprise at least a part of the amino acid sequence shown in Fig. 2.

Of course, functional derivatives as well as fragments of the polypeptides according to the invention are also included in the present invention. Functional derivatives are meant to include polypeptides in which one or more of the amino acids have been replaced with a chemically comparable one.

The crux is of course that they should still show human ZP3 antigenicity. This means that upon administration (possibly with an adjuvant) they should give rise to antibodies which recognize human ZP3.

The polypeptides according to the invention can be produced either synthetically or by recombinant DNA technology. Methods for producing synthetic polypeptides are well known in the art and do not need any further elaboration.

Production of polypeptides by recombinant DNA techniques is a general method which is known, but which has a lot of possibilities all leading to somewhat different results. The polypeptide to be expressed is coded for by a DNA sequence or more accurately by a nucleic acid sequence.

This nucleic acid sequence must be transcribed (optionally) and translated to the wanted polypeptide. In order to reach that goal the nucleic acid sequence is normally cloned into a vector with which a host cell is transformed. The vector can be either self replicating or it may integrate into the DNA of the host.

Different host cells lead to different polypeptides. Prokaryotes do not possess the organelles necessary for glycosylation. The polypeptides produced by them will be without carbohydrate side chains. Eukaryotes do have the glycosylation machinery, but yeast cells will give a different glycosylation pattern than mammalian cells.

Preferred for the polypeptides according to the invention is an expression system which gives the most "natural" glycosylation pattern. Towards this end mammalian cells are most preferred.

We have also identified epitopes on the human ZP3 polypeptide which may have contraceptive potential. These epitopes comprise at least a part of one of the following sequences:

-ThrLeuMetValMetValSerLys-,

-SerArgArgGlnProHisValMetSerGln-,

-GluValGlyLeuHisGluCysGlyAsnSerMetGlnValThrAspAspAlaLeu-,

-PheSerLeuArgLeuMetGluGluAsnTrpAsnAlaGluLysArgSerProThrPhe-,

-CysGlyThrProSerHisSerArgArgGlnProHisValMetSerGlnTrpSer-,

or

-SerGlnTrpSerThrSerAlaSerArgAsnArgArgHisValThrGluGlu
AlaAspValCysValGlyAlaThrAspLeuProGlyGlnGluTrp-.

Small polypeptides like these are more easily produced synthetically. They can also be linked together with the same or different "epitopes" to form a larger more antigenic polypeptide.

One aim of all the polypeptides according to the invention is to produce a contraceptive vaccine using them. The vaccination may be either a passive or an active immunization.

For an active immunization a polypeptide according to the invention is administered to a female (possibly with an adjuvant). The administration will give rise to an immune response by the female. Antibodies will be produced which recognize human ZP3 on the ovum. These antibodies will specifically bind to the sperm receptor binding site so that spermatozoa cannot bind, or otherwise the antibodies will prevent this binding through steric hindrance. Passive immunization basically works the same way. Instead of the antigen or its mimicry the antibodies against it are directly administered. Thus, antibodies have to be raised against the polypeptides according to the invention.

This is achieved through an active immunization scheme of a suitable mammal. The B-lymphocytes of the mammal are harvested after a suitable period of time and immortalized through fusion or transformation. These methods are well known in the art. Antibodies can be isolated from the culture of the immortalized lymphocytes.

There is, however, a problem with antibodies of animal origin. Upon repeated administration they will give rise to an anti-antibody response in the immunized woman. It is therefore preferred to use humanized antibodies or small parts of antibodies which will not lead to an immune response. Methods for humanizing antibodies, such as CDR-grafting, are known (Jones et al., Nature 321, 522-525, 1986). Methods for producing fragments of antibodies which are still specific for the antigen of the original antibody are also known (Udaka et al., Molec. Immunol. 27, 25-35; 1990). Another possibility to avoid antigenic response to antibodies to polypeptides according to the invention is the use of human antibodies or fragments or derivatives thereof.

Human antibodies can be produced by in vitro stimulation of isolated B-lymphocytes, or they can be isolated from (immortalized) B-lymphocytes which have been harvested from a female immunized with at least one polypeptide according to the invention. Another object of the invention is the use of ZP3 polypeptide and antibodies directed to it in diagnostic test kits.

Some antibodies to human ZP3 have now been obtained. It lies within the skill of the art to produce anti-idiotype antibodies which recognize the antigen binding site of the antibody and therefore are an "internal image" of the antigen.

These anti-idiotype antibodies will be also very useful for vaccine and diagnostic purposes.

In the following experimental part the isolation of the human ZP3 DNA-sequence and the human ZP3 amino acid sequence, as well as a method of expressing a recombinant polypeptide showing ZP3 antigenicity, as well as a procedure to make a synthetic polypeptide showing such antigenicity are shown. These examples are merely meant to illustrate the invention and should not be constructed as a limitation of its scope.

EXAMPLES.

MATERIALS AND METHODS.

All recombinant DNA procedures were performed essentially according to procedures known as such (Sambrook et al., Molecular Cloning, a laboratory manual, 2nd edition, CSH-laboratory press, 1989).

Restriction enzymes and DNA modifying enzymes were used as recommended by the suppliers.

A human ovary gt10 cDNA library was purchased from Stratagene.

Oligonucleotide synthesis.

Oligonucleotides were prepared on an Applied Biosystems 381A DNA synthesizer and directly used for cloning purposes.

Peptide synthesis.

Oligopeptides were produced by solid phase peptide synthesis using procedures described by Fields and Noble (*Int. J. Pept. Prot. Res.* 35, 160-214; 1990).The following ZP3 peptides were synthesized:(amino acids are given in 3 letter code)

- ZP3(93-110):
GluValGlyLeuHisGluCysGlyAsnSerMetGlnValThrAspAspAlaLeu
- ZP3(172-190):
PheSerLeuArgLeuMetGluGluAsnTrpAsnAlaGluLysArgSerProThrPhe
- ZP3(327-344):
CysGlyThrProSerHisSerArgArgGlnProHisValMetSerGlnTrpSer
- ZP3(341-360):
SerGlnTrpSerThrSerAlaSerArgAsnArgArgHisValThrGluGluAlaAspVal
- ZP3(362-372):
CysValGlyAlaThrAspLeuProGlyGlnGluTrp

Antibody production.

Polyclonal antisera were raised with various antigens:

- **Total human zona pellucida.** Salt stored human zonae pellucidae were heat solubilized and mixed with freunds adjuvants for immunization of rabbits. Antiseraum titers were assayed on ELISA plates coated with porcine ZP-proteins.

- **ßGal-ZP3 fusion protein.** The fusion protein was partially purified from sonicated bacteria based on its insolubility and separated by SDS polyacrylamide gelelectrophoresis (SDS-PAGE). By electro-blotting the proteins were transferred to a nitrocellulose membrane. The region carrying the hybrid protein was excised and dissolved in DMSO. This was mixed in a 1:1 ratio with Freunds adjuvants and used for immunization of rabbits.
- **CHO produced ZP3.** A similar procedure was followed for the ZP3 protein produced by Chinese hamster ovary (CHO) cells. Concentrated culture medium was separated by SDS-PAGE. The 40-60 kiloDalton region was excised and used for immunization of mice.
- **Oligopeptides.** Synthesized peptides were physically linked to keyhole limpet hemocyanin (KLH) and injected in rabbits. As a control rabbits were immunized with KLH only. Sera were screened on ELISA microtitre plates coated with peptide.

Human egg fluorescence assay.

Salt stored unfertilized human eggs (after 48 hour incubation with sperm in an IVF program) were incubated with antisera (1:50 diluted in buffer A [PBS + 5% BSA]). After three washes eggs were incubated with a second antibody (swine anti rabbit or mouse conjugated to FITC, 1:100 diluted in buffer A) and incubated for 1 hour at 37 ^{o}C. Following three additional wash steps fluorescence was measured with a Nikon microscope and exposure analyser. Non-stained (negative) control eggs are dark, with a long exposure time, and positively stained zonae pellucidae have shorter exposure times.

Human sperm-zona binding assay.

Human eggs (see above), were incubated in rabbit serum, washed 3 times with buffer (BWW + 3% BSA), incubated in buffer (control) or antibodies (undiluted, 37^{o}C,1hour), washed 3 times and introduced in droplets of human capacitated spermatozoa (10^{7} sperm/ml, 37 ^{o} C, 16 hour). Loosely adherent spermatozoa were removed from eggs by repeated pipetting. Bound spermatozoa were fixed and stained with BWW containing 1% glutaraldehyde and Hoechst (H33258, 20 g/ml) and the number of bound spermatozoa were counted with a fluorescence microscope.

RESULTS.

Synthesis and construction of a mouse ZP3 probe.

A 135 bp mouse ZP3 probe was constructed by assembling 8 synthetic oligonucleotides (27-51 mers). This fragment, comprising part of exon 5 and 6 of the murine gene (position 771 to 909 in Ringuette et al., Developmental Biology 127, 287-295; 1988) was provided with unique 5' BamHI and 3' HindIII restriction sites and subcloned in pGEM3 (Promega). Subsequently its nucleotide sequence was verified.

Cloning and characterization of the human ZP3 gene.

A human genomic EMBL3 library was screened with a ^{32}P-labeled ZP3 probe (135 bp fragment described above). Three overlapping EMBL clones showed a strong hybridization and were characterized in more detail. A limited physical map of these clones is schematically depicted in Fig. 1. Restriction fragments from clones I1 and D1 were subcloned in pGEM vectors and M13mp18/19 and used for genomic characterization of the human gene. This involved localization of the exons by Southern blot analysis with ^{32}P-labeled oligonucleotides from the mouse ZP3 sequence followed by sequence analysis. As is demonstrated in Fig. 1, the human gene consists of 8 exons spread over approximately 20 kb of genomic DNA. All identified exons are flanked by consensus splice donor and splice acceptor signals (not shown). From sequence analysis of all exons the complete coding sequence for the ZP3 gene could be deduced (presented in Fig. 2). The human ZP3 gene encodes a polypeptide of 372 amino acids with a calculated molecular weight of 41437 Dalton.

Cloning of human ZP3 cDNA.

A human ovary cDNA library (gt10) was screened with ^{32}P-labeled fragments from human ZP3 exon 1, 5 and 7. This yielded several partial cDNA's that contained only the 3' half of the ZP3 coding sequence (exon 5 to 8).

Sequence analysis of 3 independent cDNA clones confirmed the previously determined genomic ZP3 sequence except for one residue in exon 7 (see Fig. 2). At nucleotide position 1064 a G and C residue was found in genomic and cDNA respectively. In the encoded amino acid sequence this yields an arginine or a threonine residue respectively at position 345. This sequence difference probably represents a polymorphism in the human ZP3 gene and polypeptide.

Expression of recombinant ZP3 in CHO cells.

For expression in CHO cells the size of the ZP3 gene has been reduced to allow insertion in a mammalian expression vector. Various ZP3 DNA fragments have been assembled to a 'minigene' as is illustrated in Fig. 3. Exon 1 and 2 were joined by PCR-technology (Horton et al., Gene 77, 61-68, 1989; Yon and Fried, Nucl. Acids Res. 17, 4895, 1989)) and provided with 5' EcoRI and 3' XbaI sites respectively. Subsequently, this DNA fragment was ligated to a genomic XbaI-SalI fragment carrying exon 3, 4 and part of exon 5 and the partial cDNA harboring exon 5 to 8 on a SalI-HindIII fragment. The resulting 2.7 kb 'minigene' contains a truncated intron between exon 2 and 3 and the natural introns between ZP3 exons 3 and 4 and exon 4 and 5. The integrity of the polypeptide encoding nucleotide information of this ZP3 construct was completely verified by sequence analysis.

The ZP3 'minigene' was subsequently inserted in a mammalian expression vector in which the ZP3 gene is driven by the strong SV40 early promoter. In addition, this vector harbored ß-Globin splicing and SV40 poly-adenylation signals for correct RNA processing of the expressed gene and the selectable marker gene aminoglycosylphosphotransferase (Colbere-Garapin et al., J. Mol. Biol. 150, 1-14, 1981) which allows for isolation of stable transformants by selection for G418 resistance. CHO cells were transfected with the ZP3 expression construct using the calcium-phosphate precipitation technique (Graham and van der Eb, Virology 52, 456-467, 1973; Wigler et al., Proc. Natl. Acad. Sci USA 76,1373-1376, 1979). Mass populations of G418 resistant transformants (representing 300-500 independent clones) were analysed for expression of the recombinant ZP3 gene.

As is demonstrated in Fig. 4, Northern blot analysis of total RNA from pooled transformants with a ^{32}P-labeled ZP3 probe reveals a relatively high level of expression of the transfected ZP3 gene (as compared to the highly expressed actin gene, not shown). Moreover, it was demonstrated that the minigene RNA is correctly processed to a mRNA which is slightly larger then the natural transcript found in human ovary RNA (see Fig. 4). This size difference is most likely due to flanking 5' and 3' sequences present in the expression vector.

Correct splicing and processing of the recombinant gene was further confirmed by isolation of a partial cDNA carrying exon 1 to 5 from G418 resistant CHO transformants. Sequence data of this cDNA were similar to the transfected DNA and support the conclusion that the introns are correctly spliced from the ZP3 minigene transcript.

Culture media of growing mass populations were examined for the presence of recombinant ZP3 polypeptide by Western blot analysis. With a polyclonal antiserum directed against deglycosylated porcine ZP3 (Henderson et al., Gamete Research 18, 251-265, 1987) recombinant ZP3 polypeptide could be detected in culture medium. This signal was absent in medium from non-transfected CHO cells.

Expression of recombinant ZP3 in E. coli.

For expression in E.coli first a full lenght ZP3 cDNA was constructed using a cDNA fragment covering exon 1 to 5 isolated by PCR from CHO transformants and the partial cDNA clone (harboring exon 5 to 8) isolated from a human ovary cDNA library. Four ZP3 cDNA fragments were cloned in frame to the E.coli LacZ gene in pEX-plasmids (Biores) allowing the production of a ß-galactosidase-ZP3 fusion polypeptide as reported by Stanley and Luzio, EMBO J. 3, 1429-1434, 1984). The expression of fusion polypeptides in E.coli was examined by SDS polyacrylamide gelelectrophoresis (SDS-PAGE) and western blot analysis. The results of these experiments are presented in Fig. 5. Following heat shock induction, all four LacZ-ZP3 constructs yielded fusion polypeptides , although to a much lesser extend then the LacZ gene. The molecular weight of the hybrid polypeptides, as observed on coomassie stained gels (see Fig. 5A), were in agreement with the expressed ZP3 parts. This was further confirmed by western blot analysis with anti-ß-galactosidase antibodies (not shown) and a rabbit antiserum raised against ZP3 amino acids 341 to 360 coupled to KLH

(Fig. 5B). Only fusion polypeptides carrying this region, i.e. ZP3-A and ZP3-D, were recognized by the antiserum. An antiserum against KLH yielded a result similar to the background staining in Fig. 5B (not shown). In addition, ßGal-ZP3 fusion polypeptides were recognized by antisera raised against total human zonae pellucidae (not shown).

Binding of ZP3 antibodies to human eggs.

A human egg fluorescence assay was developed to characterize the binding of the various ZP antibodies to human zonae pellucidae. The results of three different experiments are depicted in Fig. 6. Serum against KLH and a normal mouse serum failed to give fluorescent staining whereas with all antisera against ZP components strong to intermediate levels of fluorescence were observed. From the presented data it can be inferred that antisera against ZP3(93-110), ZP3(172-190), ZP3(327-344), ZP3(341-360) and ZP3(362-372) recognize human oocytes.

Effect of ZP antibodies on sperm-egg recognition.

The contraceptive potential of the ZP3 antibodies was analysed in a human sperm-zona binding assay. The results of this assay is presented in Fig. 7. Antibodies against total human zonae pellucidae showed 75% inhibition of sperm-zona binding. An antiserum against KLH does not interfere with sperm-egg binding. In contrast, a mixture of ZP3-peptide antibodies (peptides: ZP3(93-110), ZP3(172-190), ZP3(341-360) and ZP3(362-372)) showed a relative inhibition of sperm binding of 35%. Antibodies against recombinant ZP3 from CHO cells reduced sperm binding with 55%.

Legends to the Figures.

Figure 1.
Restriction maps of EMBL clones I1, D1 and E1 (A) and genomic organization of the human ZP3 gene. Exons are indicated by black boxes. Restriction sites: S, SalI; B, BamHI.

Figure 2.
Nucleotide and amino acid sequence of human ZP3. At position 1064 a G or C residue was found in genomic and cDNA respectively. This apparent polymorphism yields a Thr or Arg amino acid residue. Arrows indicate exon junctions. Exon number is indicated in circles. The polyadenylation signal is underlined.

Figure 3.
Size reduction of ZP3 gene for insertion in a mammalian expression vector. A 2.7 kb ZP3 'minigene' was assembled from the following three fragments: *A*, exon 1 and exon 2 joined in frame to each other by PCR-technology yielding a EcoRI-XbaI fragment; *B*, a genomic XbaI-SalI fragment with exon 3, 4 and part of exon 5; *C*, a SalI-HindIII ZP3 cDNA fragment with exon 5 to 8 (provided with artificial 3' BamHI and HindIII sites). The three fragments were cloned in a pGEM vector (Promega) opened with EcoRI and HindIII and subsequently as a 2.7 kb EcoRI-BamHI fragment placed behind the SV40 promoter in a mammalian expression vector.

Figure 4.
Detection of ZP3 mRNA in total RNA (15 g) from CHO mass populations transfected with the ZP3 DNA and poly(A)$^+$ RNA (5 g) from a human ovary. Lane 1, CHO; lane 2, CHO ZP3 transformants; lane 3, human ovary. Total ZP3 cDNA in pGEM3 was used as a probe.

Figure 5.
Production of ßGal-ZP3 fusion polypeptides in E.coli strain POP2136 carrying pEX-plasmids with human ZP3 cDNA inserts.

A. Coomassie staining of polyacrylamide gel (10%) and *B.* Western blot analysis with anti-ZP3-341-360 antiserum (1:150 dilution). From left to right the following samples are loaded on the gels (-, before induction; + 2 hours after induction): control pEX; pEX-ZP3A (KpnI-BamHI fragment; total cDNA); pEX-ZP3B (KpnI-SalI fragment; cDNA truncated at exon 5 encoding amino acid 1-241); pEX-ZP3C (KpnI-SphI fragment; cDNA truncated at end of exon 1 encoding amino acid 1-102); pEX-ZP3D (SmaI-BamHI fragment; 3' half of exon 5 + exon 6,7 and 8 encoding amino acid 257-372). Molecular weight (M) markers are at the extreme left (indicated in kiloDalton). Bacteria were grown at 30° C till OD_{600} = 0.5-1 and subsequently induced for two hours at 42 °C. Aliquots were taken, centrifuged and total cells lysates were analysed.

Figure 6.

Binding of different antibodies (see materials and methods) to human eggs. Amount of second antibody labeled with FITC was measured with an exposure analyser and expressed as percentage of control (100% = dark, 0% = fluorescent eggs). 1: control; 2: antiserum against human zonae pellucidae; 3: antiserum against KLH; 4-8: antisera against ZP3(93-110), ZP3(172-190), ZP3(327-344), ZP3(341-360) and ZP3(362-372); 9: antiserum against ßGal-ZP3; 10:mixture of sera of 3 mice immunized with recombinant CHO produced ZP3; 11: mixture of sera of 3 control mice. Average exposure time as percentage of control and standard error of the mean are given.

Figure 7.

Inhibition of sperm-zona binding by anti-ZP3 antibodies (see materials and methods). 1: control; 2: antiserum against KLH; 3: antiserum against human zonae pellucidae; 4: mixture of antisera against ZP3(93-110), ZP3(172-190), ZP3(341-360) and ZP3(362-372); 5: mixture of sera of 3 mice immunized with recombinant CHO produced ZP3. Average number of bound spermatozoa on total zonae pellucidae and standard error of the mean are given.

CLAIMS

1) Pure polypeptide or a functional derivative thereof having human ZP3 activity or having, at least in part, human ZP3 antigenicity.

2) Polypeptide according to claim 1, characterized in that it is produced recombinantly.

3) Polypeptide according to claim 1 or 2, characterized in that it comprises at least part of the following sequence:

```
  1
MetGluLeuSerTyrArgLeuPheIleCysLeuLeuLeuTrpGlySerThrGluLeuCys

 21
TyrProGlnProLeuTrpLeuLeuGlnGlyGlyAlaSerHisProGluThrSerValGln

 41
ProValLeuValGluCysGlnGluAlaThrLeuMetValMetValSerLysAspLeuPhe

 61
GlyThrGlyLysLeuIleArgAlaAlaAspLeuThrLeuGlyProGluAlaCysGluPro

 81
LeuValSerMetAspThrGluAspValValArgPheGluValGlyLeuHisGluCysGly

101
AsnSerMetGlnValThrAspAspAlaLeuValTyrSerThrPheLeuLeuHisAspPro

121
ArgProValGlyAsnLeuSerIleValArgThrAsnArgAlaGluIleProIleGluCys

141
ArgTyrProArgGlnGlyAsnValSerSerGlnAlaIleLeuProThrTrpLeuProPhe

161
ArgThrThrValPheSerGluGluLysLeuThrPheSerLeuArgLeuMetGluGluAsn

181
TrpAsnAlaGluLysArgSerProThrPheHisLeuGlyAspAlaAlaHisLeuGlnAla

201
GluIleHisThrGlySerHisValProLeuArgLeuPheValAspHisCysValAlaThr

221
ProThrProAspGlnAsnAlaSerProTyrHisThrIleValAspPheHisGlyCysLeu
```

241
ValAspGlyLeuThrAspAlaSerSerAlaPheLysValProArgProGlyProAspThr

261
LeuGlnPheThrValAspValPheHisPheAlaAsnAspSerArgAsnMetIleTyrIle

281
ThrCysHisLeuLysValThrLeuAlaGluGlnAspProAspGluLeuAsnLysAlaCys

301
SerPheSerLysProSerAsnSerTrpPheProValGluGlyProAlaAspIleCysGln

321
CysCysAsnLysGlyAspCysGlyThrProSerHisSerArgArgGlnProHisValMet

341
SerGlnTrpSerThrSerAlaSerArgAsnArgArgHisValThrGluGluAlaAspVal

361
ThrValGlyAlaThrAspLeuProGlyGlnGluTrp

4) Polypeptide according to claim 1, 2 or 3, characterized in that it is at least partially glycosylated.

5) Polypeptide according to claim 3 or 4, characterized in that it comprises at least one of the following sequences:

-ThrLeuMetValMetValSerLys-,

-SerArgArgGlnProHisValMetSerGln-,

-GluValGlyLeuHisGluCysGlyAsnSerMetGlnValThrAspAspAlaLeu-,

-PheSerLeuArgLeuMetGluGluAsnTrpAsnAlaGluLysArgSerProThrPhe-,

-CysGlyThrProSerHisSerArgArgGlnProHisValMetSerGlnTrpSer-,

or

-SerGlnTrpSerThrSerAlaSerArgAsnArgArgHisValThrGluGlu
AlaAspValCysValGlyAlaThrAspLeuProGlyGlnGluTrp-.

6) Polypeptide having human ZP3 activity or having, at least in part, human ZP3 antigenicity, characterized in that it comprises at least part of the following sequence:

-SerGlnTrpSerThrSerAlaSerArgAsnArgArgHisValThrGluGluAlaAspVal-

7) Nucleic acid sequence coding for at least part of a polypeptide according to claims 1-6.

8) Nucleic acid sequence according to claim 7, characterized in that it comprises at least a part of the following sequence or functional derivatives thereof:

```
         10         20         30         40         50         60
GAGGCGGCTGCCTGCTGCTCTGCAGGTACCATGGAGCTGAGCTATAGGCTCTTCATCTGC

         70         80         90        100        110        120
CTCCTGCTCTGGGGTAGTACTGAGCTGTGCTACCCCCAACCCCTCTGGCTCTTGCAGGGT

        130        140        150        160        170        180
GGAGCCAGCCATCCTGAGACGTCCGTACAGCCCGTACTGGTGGAGTGTCAGGAGGCCACT

        190        200        210        220        230        240
CTGATGGTCATGGTCAGCAAAGACCTTTTTGGCACCGGGAAGCTCATCAGGGCTGCTGAC

        250        260        270        280        290        300
CTCACCTTGGGCCCAGAGGCCTGTGAGCCTCTGGTCTCCATGGACACAGAAGATGTGGTC

        310        320        330        340        350        360
AGGTTTGAGGTTGGACTCCACGAGTGTGGCAACAGCATGCAGGTAACTGACGATGCCCTG

        370        380        390        400        410        420
GTGTACAGCACCTTCCTGCTCCATGACCCCCGCCCCGTGGGAAACCTGTCCATCGTGAGG

        430        440        450        460        470        480
ACTAACCGCGCAGAGATTCCCATCGAGTGCCGCTACCCCAGGCAGGGCAATGTGAGCAGC

        490        500        510        520        530        540
CAGGCCATCCTGCCCACCTGGTTGCCCTTCAGGACCACGGTGTTCTCAGAGGAGAAGCTG

        550        560        570        580        590        600
ACTTTCTCTCTGCGTCTGATGGAGGAGAACTGGAACGCTGAGAAGAGGTCCCCCACCTTC

        610        620        630        640        650        660
CACCTGGGAGATGCAGCCCACCTCCAGGCAGAAATCCACACTGGCAGCCACGTGCCACTG
```

```
        670        680        690        700        710        720
CGGTTGTTTGTGGACCACTGCGTGGCCACACCGACACCAGACCAGAATGCCTCCCCTTAT

        730        740        750        760        770        780
CACACCATCGTGGACTTCCATGGCTGTCTTGTCGACGGTCTCACTGATGCCTCTTCTGCA

        790        800        810        820        830        840
TTCAAAGTTCCTCGACCCGGGCCAGATACACTCCAGTTCACAGTGGATGTCTTCCACTTT

        850        860        870        880        890        900
GCTAATGACTCCAGAAACATGATATACATCACCTGCCACCTGAAGGTCACCCTAGCTGAG

        910        920        930        940        950        960
CAGGACCCAGATGAACTCAACAAGGCCTGTTCCTTCAGCAAGCCTTCCAACAGCTGGTTC

        970        980        990       1000       1010       1020
CCAGTGGAAGGCCCGGCTGACATCTGTCAATGCTGTAACAAAGGTGACTGTGGCACTCCA

       1030       1040       1050       1060       1070       1080
AGCCATTCCAGGAGGCAGCCTCATGTCATGAGCCAGTGGTCCACGTCTGCTTCCCGTAAC

       1090       1100       1110       1120       1130       1140
CGCAGGCATGTGACAGAAGAAGCAGATGTCACCGTGGGGGCCACTGATCTTCCTGGACAG

       1150       1160       1170       1180       1190       1200
GAGTGGTGACCATGAAGTAGAGCAGTGGGCTTTGCCTTCTGACACCTCAGTGGTGCTGCT

       1210       1220       1230       1240       1250       1260
GGGCGTAGGCCTGGCTGTGGTGGTGTCCCTGACTCTGACTGCTGTTATCCTGGTTCTCAC

       1270       1280       1290       1300       1310
CAGGAGGTGTCGCACTGCCTCCCACCCTGTGTCTGCTTCCGAATAAAAGAAGAAA
```

9) A vector comprising a nucleic acid sequence according to claims 7 or 8.

10) A host cell comprising a nucleic acid sequence according to claim 9, or a sequence according to claims 7 or 8.

11) A pharmaceutical composition comprising a polypeptide according to any one of the claims 1-6.

12) An immunocontraceptive vaccine comprising a polypeptide according to any one of the claims 1-6 and an adjuvant.

13) An immunocontraceptive vaccine comprising an antibody which recognizes a polypeptide according to any one of the claims 1-6.

14) A method for producing a polypeptide according to any one of the claims 1-4, characterized in that a host cell is transformed with a vector comprising a nucleic acid sequence coding for such a polypeptide and culturing said host cell in a suitable medium and isolating the polypeptide from the culture.

15) A method for producing an antibody to a polypeptide according to any one of the claims 1-6, characterized in that a suitable animal is injected with said polypeptide and an adjuvant, and that the B-lymphocytes of said animal are harvested after a suitable period of time and that antibody producing cells are selected and immortalized, after which they are cultured and the antibodies are isolated from the culture.

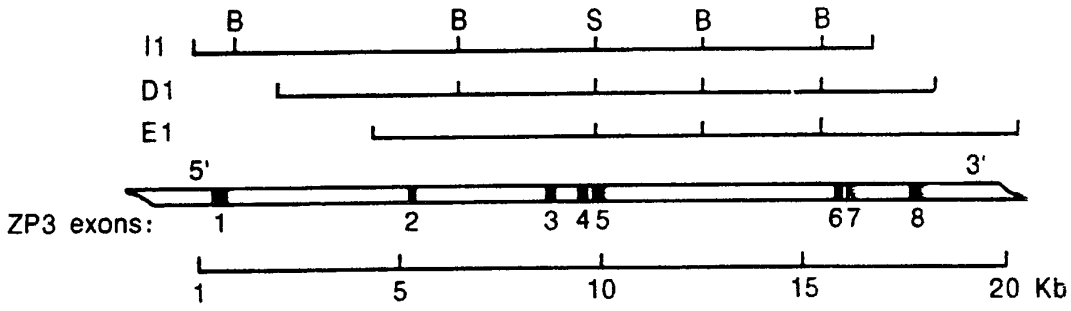


Figure 1

Fig. 2

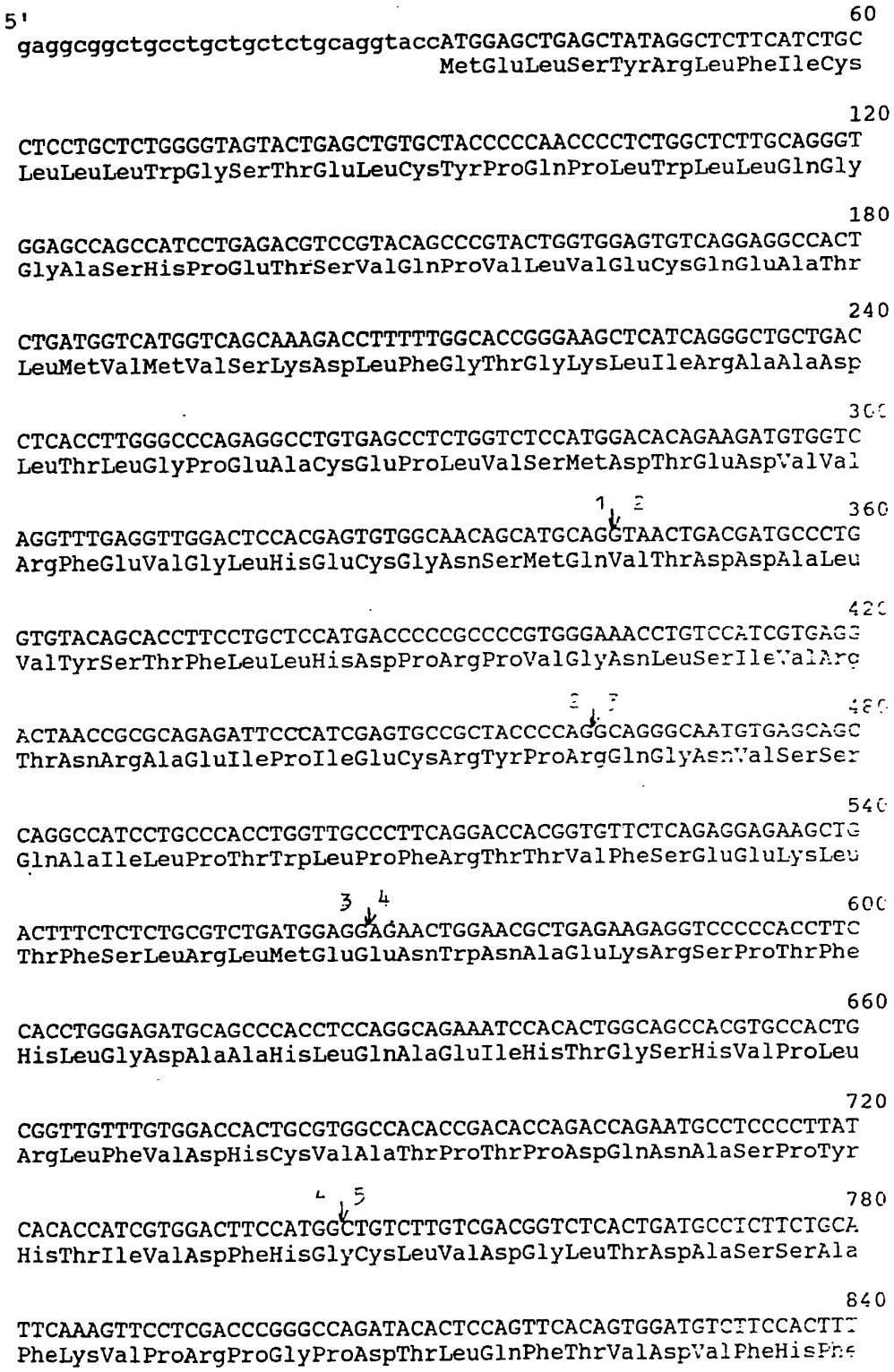

5'
60
gaggcggctgcctgctgctctgcaggtaccATGGAGCTGAGCTATAGGCTCTTCATCTGC
MetGluLeuSerTyrArgLeuPheIleCys
120
CTCCTGCTCTGGGGTAGTACTGAGCTGTGCTACCCCCAACCCCTCTGGCTCTTGCAGGGT
LeuLeuLeuTrpGlySerThrGluLeuCysTyrProGlnProLeuTrpLeuLeuGlnGly
180
GGAGCCAGCCATCCTGAGACGTCCGTACAGCCCGTACTGGTGGAGTGTCAGGAGGCCACT
GlyAlaSerHisProGluThrSerValGlnProValLeuValGluCysGlnGluAlaThr
240
CTGATGGTCATGGTCAGCAAAGACCTTTTTGGCACCGGGAAGCTCATCAGGGCTGCTGAC
LeuMetValMetValSerLysAspLeuPheGlyThrGlyLysLeuIleArgAlaAlaAsp
300
CTCACCTTGGGCCCAGAGGCCTGTGAGCCTCTGGTCTCCATGGACACAGAAGATGTGGTC
LeuThrLeuGlyProGluAlaCysGluProLeuValSerMetAspThrGluAspValVal
1 2
360
AGGTTTGAGGTTGGACTCCACGAGTGTGGCAACAGCATGCAGGTAACTGACGATGCCCTG
ArgPheGluValGlyLeuHisGluCysGlyAsnSerMetGlnValThrAspAspAlaLeu
420
GTGTACAGCACCTTCCTGCTCCATGACCCCCGCCCCGTGGGAAACCTGTCCATCGTGAGG
ValTyrSerThrPheLeuLeuHisAspProArgProValGlyAsnLeuSerIleValArg
6 7
480
ACTAACCGCGCAGAGATTCCCATCGAGTGCCGCTACCCCAGGCAGGGCAATGTGAGCAGC
ThrAsnArgAlaGluIleProIleGluCysArgTyrProArgGlnGlyAsnValSerSer
540
CAGGCCATCCTGCCCACCTGGTTGCCCTTCAGGACCACGGTGTTCTCAGAGGAGAAGCTG
GlnAlaIleLeuProThrTrpLeuProPheArgThrThrValPheSerGluGluLysLeu
3 4
600
ACTTTCTCTCTGCGTCTGATGGAGGAGAACTGGAACGCTGAGAAGAGGTCCCCCACCTTC
ThrPheSerLeuArgLeuMetGluGluAsnTrpAsnAlaGluLysArgSerProThrPhe
660
CACCTGGGAGATGCAGCCCACCTCCAGGCAGAAATCCACACTGGCAGCCACGTGCCACTG
HisLeuGlyAspAlaAlaHisLeuGlnAlaGluIleHisThrGlySerHisValProLeu
720
CGGTTGTTTGTGGACCACTGCGTGGCCACACCGACACCAGACCAGAATGCCTCCCCTTAT
ArgLeuPheValAspHisCysValAlaThrProThrProAspGlnAsnAlaSerProTyr
4 5
780
CACACCATCGTGGACTTCCATGGCTGTCTTGTCGACGGTCTCACTGATGCCTCTTCTGCA
HisThrIleValAspPheHisGlyCysLeuValAspGlyLeuThrAspAlaSerSerAla
840
TTCAAAGTTCCTCGACCCGGGCCAGATACACTCCAGTTCACAGTGGATGTCTTCCACTTT
PheLysValProArgProGlyProAspThrLeuGlnPheThrValAspValPheHisPhe

Fig. 2 (c nt.)

```
                        5  6
                        ↓
                                                                  900
GCTAATGACTCCAGAAACATGATATACATCACCTGCCACCTGAAGGTCACCCTAGCTGAG
AlaAsnAspSerArgAsnMetIleTyrIleThrCysHisLeuLysValThrLeuAlaGlu

                                                    6  7
                                                    ↓
                                                                  960
CAGGACCCAGATGAACTCAACAAGGCCTGTTCCTTCAGCAAGCCTTCCAACAGCTGGTTC
GlnAspProAspGluLeuAsnLysAlaCysSerPheSerLysProSerAsnSerTrpPhe

                                                                 1020
CCAGTGGAAGGCCCGGCTGACATCTGTCAATGCTGTAACAAAGGTGACTGTGGCACTCCA
ProValGluGlyProAlaAspIleCysGlnCysCysAsnLysGlyAspCysGlyThrPro

                                                                 1080
AGCCATTCCAGGAGGCAGCCTCATGTCATGAGCCAGTGGTCCAGGTCTGCTTCCCGTAAC
SerHisSerArgArgGlnProHisValMetSerGlnTrpSerArgSerAlaSerArgAsn

          7  8
          ↓
                                                                 1140
CGCAGGCATGTGACAGAAGAAGCAGATGTCACCGTGGGGGCCACTGATCTTCCTGGACAG
ArgArgHisValThrGluGluAlaAspValThrValGlyAlaThrAspLeuProGlyGln

                                                                 1200
GAGTGGtgaccatgaagtagagcagtgggctttgccttctgacacctcagtggtgctgct
GluTrp

                                                                 1260
gggcgtaggcctggctgtggtggtgtccctgactctgactgctgttatcctggttctcac

                                                            1314
caggaggtgtcgcactgcctcccaccctgtgtctgcttccgaataaaagaagaaa     3'
```

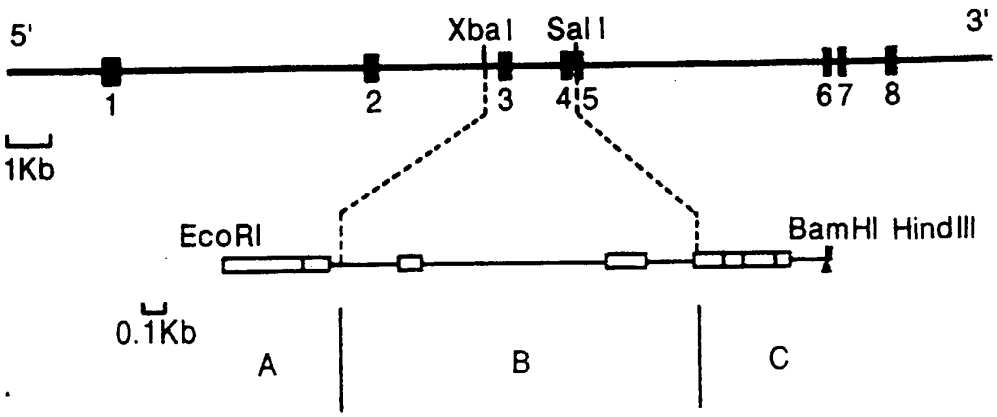


Figure 3

Fig. 4

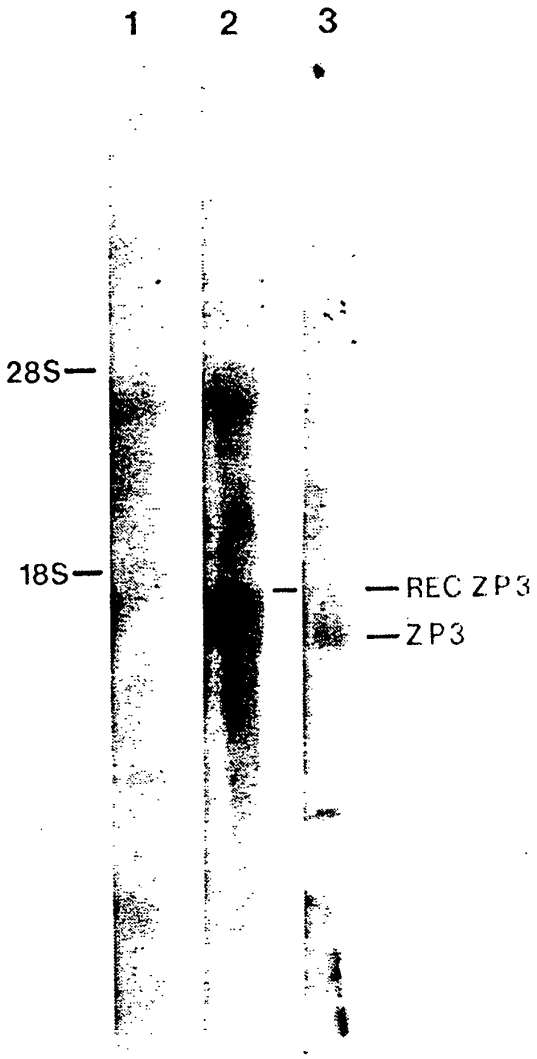
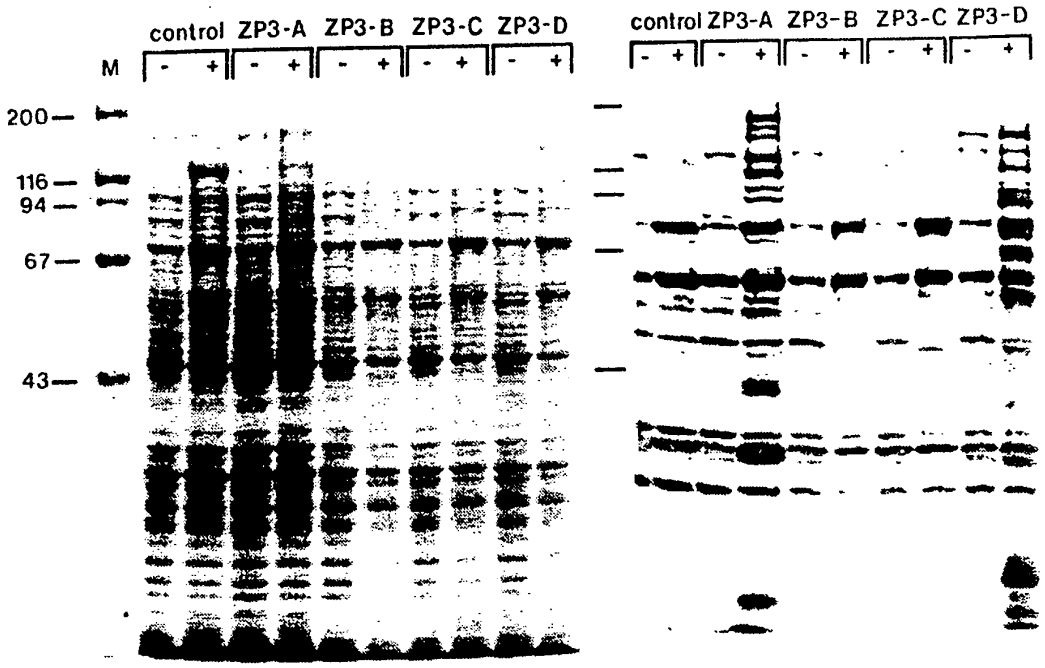


Fig. 5


control ZP3-A ZP3-B ZP3-C ZP3-D
M
- + - + - + - + - +
200
116
94
67
43
control ZP3-A ZP3-B ZP3-C ZP3-D
- + - + - + - + - +

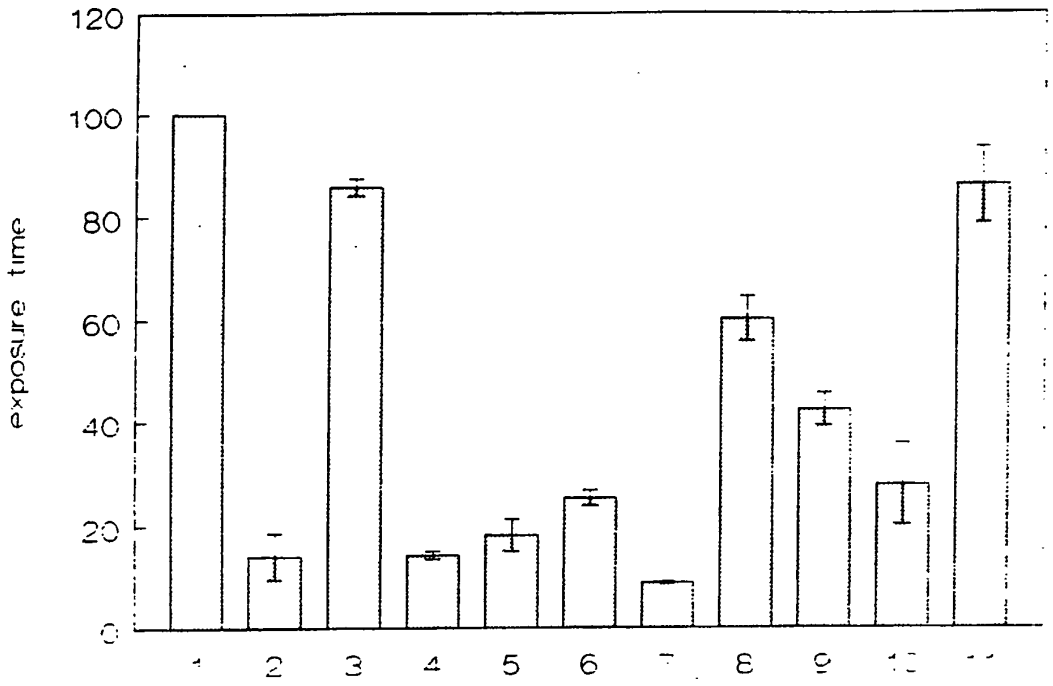


FIGURE 6

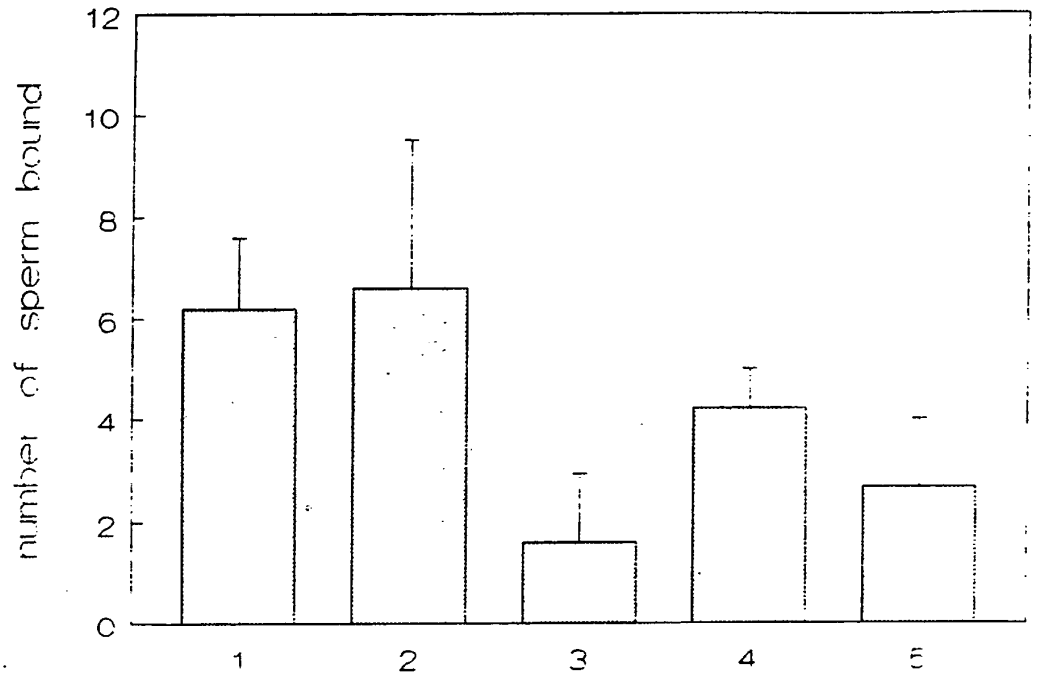


FIGURE 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/01538

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/12 C 07 K 13/00 A 61 K 39/00
C 12 N 5/16

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	PROC. NATL. ACAD. SCI. USA, vol. 87, August 1990. M.E. CHAMBERLIN et al.: "Human homolog of the mouse sperm receptor", pages 6014-6018, see the whole article ---	1-10,14
Y	WO,A,8903399 (ZONAGEN, INC.) 20 April 1989, see the whole document * Whole document * ---	1-10,14
Y	PROC. NATL. ACAD. SCI. USA, vol. 83, June 1986, pages 4341-4345; M.J. RINGUETTE et al.: "Oocyte-specific gene expression: Molecular characterization of a cDNA coding for ZP-3, the sperm receptor of the mouse zona pellucida" * Whole article * --- -/-	1-10,14

[10] Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22-10-1991	09. 01. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Nicole De Bie

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	CHEMICAL ABSTRACTS, vol. 110, no. 9, 1988, pages 425-426, abstract no. 73084e, (Columbus, Ohio, US), R.B. SHABANOWITZ et al.: "Molecular changes in the human zona pellucida associated with fertilization and human sperm-zona interactions", & ANN. N.Y. ACAD. SCI. 1988, 541, 621-32, see the whole abstract ---	1-10,14
Y	CHEMICAL ABSTRACTS, vol. 113, no. 11, 1990, pages 550-551, abstract no. 95872z, (Columbus, Ohio, US), R.B. SHABANOWITZ et al.: "Mouse antibodies to human zona pellucida: evidence that human ZP3 is strongly immunogenic and contains two distinct isomer chains", & BIOL. REPROD. 1990, 43(2), 260-70, see the whole abstract ---	1-10,14
P,Y	WO,A,9015624 (THE UNITED STATES OF AMERICA - SECRETARY US DEPARTMENT OF COMMERCE) 27 December 1990, see the whole document -----	1-10,14

FURTHER INFORMATION C NTINUED FR M THE SEC ND SHEET

V. ☐ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a)

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. Claims 1-10,14
2. Claims 11-13,15

For further information please see form PCT/ISA/206 mailed on 07.11.91.

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

   1-10,14

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101538 SA 50197

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 07/01/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8903399	20-04-89	US-A-	4996297	26-02-91
		AU-A-	2536088	02-05-89
		EP-A-	0396552	14-11-90
		JP-T-	3502571	13-06-91
WO-A- 9015624	27-12-90	AU-A-	5826790	08-01-91